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Virulence of *Rhodococcus equi* Isolates from Patients with and without AIDS

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***Rhodococcus equi* is an emerging opportunistic pathogen of human immunodeficiency virus-infected patients. Thirty-nine isolates of *R. equi* from immunocompromised patients with and without AIDS were analyzed for the presence of virulence plasmid DNA, expression of 15- to 17-kDa antigens, and their pathogenicities in mice. Of the human isolates, eight contained an 85-kb virulence plasmid, expressed 15- to 17-kDa antigens, and were virulent in mice. Nineteen isolates carried cryptic plasmids of various sizes, and the remaining 12 isolates did not contain any plasmids. These 31 isolates did not express virulence-associated antigens and were not virulent in mice. The results suggested that opportunistic infections in immunocompromised patients could be caused by both virulent and avirulent *R. equi* strains and that the pathogenesis of *R. equi* infection in immunocompromised patients appears to be different from that which occurs in foals.**

Rhodococcus equi, which causes suppurative bronchopneumonia, lymphadenitis, and enteritis in foals of less than 6 months of age, has been recognized as an important bacterial pathogen in immunocompromised patients, especially in those with AIDS (1, 6, 7, 9, 10, 12, 16, 20, 22, 26, 37). In patients with AIDS, pneumonia and lung abscesses are the most common manifestations of human immunodeficiency virus infection, which is similar to pulmonary tuberculosis (9, 23, 37). Since *Rhodococcus* species are ubiquitous in the environment (2, 23), it is likely that many cases of *Rhodococcus* infection are overlooked as contaminants in the laboratory, especially when they appear in mixed cultures (5, 37).

The recent interest in the virulence mechanisms of *R. equi* has undoubtedly been stimulated by the discovery of virulence plasmids (34–36). We have demonstrated the association of large plasmids (85- and 90-kb virulence plasmids) and 15- to 17-kDa antigens with the virulence of *R. equi*; strains showing 15- to 17-kDa antigens contained a large plasmid and were virulent in mice, whereas mutants of these strains, which were cured of the large plasmid, lacked the antigens and showed a loss of virulence (34). Recent data on the 15- to 17-kDa antigens, which are thermoregulated and expressed on the cell surface (29), suggest their possible significance as virulence factors. More recently, the genetic region responsible for the 15- to 17-kDa antigens has been determined to be in the virulence plasmid (13).

However, little is known about the characteristics of *R. equi* isolates from humans. In the present study, we investigated plasmid and protein profiles and the pathogenicities in mice of *R. equi* clinical isolates from immunocompromised patients with and without AIDS.

MATERIALS AND METHODS

Bacterial strains. The clinical isolates of *R. equi* used in the present study are listed in Table 1. All the isolates from patients with and without AIDS were identified and were previously described in detail (1, 4–10, 12, 15, 18, 20–22, 26, 37). Strains ATCC 33701 and L1 were used as reference strains, since some of their plasmid characteristics and virulence levels have already been described (34, 35). All isolates were stored frozen with 20% glycerol in small aliquots at −80°C until use.

Gel electrophoresis and immunoblot analysis. Sodium dodecyl sulfate (SDS)-gel electrophoresis and immunoblotting analysis were performed as described previously (14, 31). Monoclonal antibody 10G5 (28) against the virulence-associated 15- to 17-kDa antigens was used for the immunoblotting procedures.

Isolation of plasmid DNA. Plasmid DNA was isolated from *R. equi* by an alkaline lysis method (3), with some modifications as described previously (34). Samples of the plasmid preparations were separated in 0.7% agarose gels at approximately 5 V/cm for 2 h.

For preparation of probe DNA, the plasmid pREAT701 (35), which was prepared by large-scale isolation, was purified by cesium chloride-ethidium bromide density gradient centrifugation (34).

Preparation of probe DNA. Plasmid DNA was labeled by the random hexanucleotide priming method with digoxigenin-dUTP (DNA labeling and detection kit, nonradioactive; Boehringer Mannheim-Yamanouchi, Tokyo, Japan).

Hybridization technique. Plasmid DNA was transferred to a sheet of nylon membrane (Nytran; Schleicher & Schuell, Inc., Keene, N.H.) by using the vacuum transfer method with VacuGene (Pharmacia-LKB Biotechnology AB, Uppsala, Sweden), without depurination, following the instructions of the manufacturer. Prehybridization and hybridization were carried out at 68°C for 4 h and 16 to 20 h, respectively, with the hybridization buffer in the commercial kit. After hybridization, the sheets were washed twice at room temperature for 5 min in 2× saline sodium citrate (SSC; 1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.2]) containing 0.1% SDS; this was followed by two 15-min washes at 68°C in 0.1× SSC containing 0.1% SDS. Immunological detection of hybridized digoxigenin-labeled probe was performed with a commercial kit.

Mouse pathogenicity test. The virulences of the clinical

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TABLE 1. Sources, references, plasmids and mouse pathogenicity of *Rhodococcus equi* isolates

Laboratory no.	Isolate	Source of bacteria	Reference or source	Plasmid (kb)[a]	Mouse pathogenicity
1	JMM	Knee wound[b]	18	—[c]	–[d]
2	1	AIDS	5	Cryptic (69)	–
3	2	AIDS	5	Cryptic (69)	–
4	3	AIDS	5	Cryptic (69)	–
5	4	AIDS	5	Cryptic (110)	–
6	5	AIDS	5	Virulence (85)	+[e]
7	6	AIDS	5	Cryptic (110)	–
8	7	Baby M[b]	5	Cryptic (56)	–
9	1	AIDS	26	Virulence (85)	+
10	2	Diabetes[b]	Joan Arnal	—	–
11	89B 68'/5	AIDS	Brian Dwyer	Cryptic (89)	–
12	89B 6386	AIDS	Brian Dwyer	Cryptic (89)	–
13	89B 6853	AIDS	Brian Dwyer	Cryptic (89)	–
14	91B 3348	AIDS	Brian Dwyer	—	–
15	91B 3348	AIDS	Brian Dwyer	Cryptic (110)	–
16	Isolate	AIDS	Jerry Smilack	Cryptic (69)	–
17	Isolate	Eye infection[b]	8	—	–
18	25852/85	Leg ulcer[b]	4	Cryptic (89)	–
19	R9930	AIDS	22	—	–
20	641	AIDS	L. Tocalli	Cryptic (110)	–
21	419	AIDS	L. Tocalli	Virulence (85)	+
22	281	AIDS	L. Tocalli	Cryptic (110)	–
23	NA	AIDS	7	Virulence (85)	+
24	SE	AIDS	7	Virulence (85)	+
25	Isolate	Renal transplant[b]	21	—	–
26	IA3	AIDS	9	Virulence (85)	+
27	AK81FOS	Lymphosarcoma[b]	12	—	–
28	WN85SAM	Pneumonia[b]	12	Virulence (85)	+
29	W4828	AIDS	15	Virulence (85)	+
30	W4880	AIDS	15	—	–
31	W4888	AIDS	15	Cryptic (23)	–
32	W4882	Non-AIDS	15	Cryptic (88)	–
33	W4900	Non-AIDS	15	Cryptic (40)	–
34	E-405	AIDS	1	—	–
35	H-7625-G	AIDS	1	—	–
36	PN1002	AIDS	20	Cryptic (110)	–
37	P3684	AIDS	16	—	–
38	D256	AIDS	6	—	–
39	22/10	AIDS	37	Cryptic (120)	–

[a] Plasmid sizes were estimated by observing migration in the gels as described by Rochelle et al. (25).
[b] The Patients did not have AIDS.
[c] —, no plasmids were detected.
[d] Avirulent.
[e] Virulent.

isolates were examined by the mouse pathogenicity test as described previously (31, 35).

RESULTS

Figure 1 shows a part of the results of plasmid profiles, immunoblotting, and Southern hybridization. As shown in Fig. 1A, the isolates tested were found to carry plasmids whose sizes were very similar to those of the virulence plasmids pREAT701 (86.1 kb) and pREL1 (89.6 kb), which were used as references (13, 35). Since it was difficult to identify a virulence plasmid by measuring electrophoretic mobilities, these isolates were then tested for the presence of 15- to 17-kDa antigens by immunoblotting with monoclonal antibody 10G5 (28) and the presence of a virulence plasmid by Southern hybridization with the pREAT701 probe (35). Among the eight isolates (Fig. 1), two were found to express 15- to 17-kDa antigens (Fig. 1b), and the plasmid DNAs of these two isolates hybridized with the pREAT701 probe (Fig. 1c), indicating that these two isolates carried the virulence plasmid. The remaining 31 isolates were also tested by agarose gel electrophoresis and then immunoblotting and Southern hybridization (data not shown). Among 31 isolates tested, 6 appeared to carry a virulence plasmid, 13 isolates carried cryptic plasmids, and 12 isolates did not carry any plasmid (Table 1).

The pathogenicities of the 39 isolates were tested in mice. Only the eight isolates carrying the virulence plasmid were found to be virulent (Table 1).

DISCUSSION

The present study revealed that the majority of clinical isolates of *R. equi* from patients with and without AIDS did not carry the virulence plasmid and were avirulent in mice. On the other hand, our recent study (35) has shown that almost all the clinical isolates of *R. equi* from infected foals contain a virulence plasmid and are virulent in mice. Moreover, in our recent study of experimental infections in foals (27), strain ATCC 33701 caused severe lesions in foals

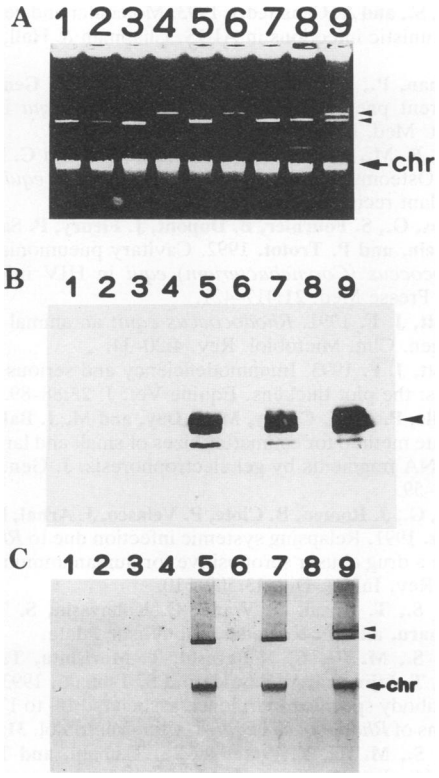

FIG. 1. (A) Plasmid profiles of *R. equi* isolates. Plasmid DNA was extracted, resolved in a 0.7% (wt/vol) agarose gel, and stained with ethidium bromide. Lanes: 1, isolate 2; 2, isolate 3; 3, isolate 4; 4, isolate 5; 5, isolate 6; 6, isolate 7; 7, isolate 9; 8, isolate 11; 9, strains ATCC 33701 and L1. The arrow on the right indicates chromosomal DNA. The arrowheads on the right indicate plasmids ATCC 33701 and L1. (B) Immunoblot profiles of *R. equi* isolates. Whole-cell preparations were analyzed by immunoblotting with monoclonal antibody 10G5. The immunoblot of the gel corresponded to the gel stained with ethidium bromide in panel A. The arrowhead on the left indicates 15- to 17-kDa antigens. (C) Southern hybridization of digoxigenin-labeled plasmid ATCC 33701 DNA probes to the DNAs of *R. equi* isolates. The Southern blot of the gel corresponds to the gel stained with ethidium bromide in panel A.

following aerosolization, but a plasmid-cured derivative of strain ATCC 33701 failed to induce any lesions. The details of the pathogenesis of *R. equi* infections in foals remain unclear except that the 15- to 17-kDa antigens are very important virulence factors (27, 29, 31, 34, 35). The pathogenesis of *R. equi* infections in immunocompromised patients with AIDS might be attributed to the fact that the organism is an intracellular pathogen, since *R. equi* contains mycolic acids in the cell wall (2, 11, 23) and induces tuberculosis-like pulmonary lesions in patients (7, 37). Gotoh et al. (11) showed that the carbon chain length of the mycolic acid of *R. equi* is critical in granuloma formation in the murine liver. The mycolic acid content and type might be more important in the intracellular survival of the organism and in the formation of granulomatous lesions in *R. equi* infections in humans. These results suggested that the pathogenesis of *R. equi* infections in immunocompromised hosts appears to be different from that which occurs in foals.

In humans, *R. equi* is not usually considered a pathogen (23). Virtually all infected patients described in this report had underlying immunosuppression, such as lymphoproliferative malignancies, renal transplants, or AIDS (1, 4–10, 12, 15, 22, 26, 37). Some of the patients without AIDS had been receiving immunosuppressive therapy (12, 21). In patients with AIDS, the inability to generate bactericidal macrophages might be due to the depletion of T-helper lymphocytes or to direct infection of macrophages with human immunodeficiency virus (19). These predisposing factors appear to increase susceptibility to opportunistic infections caused by avirulent *R. equi* strains.

In foals, *R. equi* usually causes fatal suppurative pneumonia at 1 to 2 months of age, whereas horses older than 6 months of age usually survive *R. equi* infection (2, 23, 38). The reason for the unique susceptibilities of foals by 2 months of age is still unclear, but an age-related manifestation in foals has been explained by the declining maternally derived antibody concentrations and the immaturity of cellular immune mechanisms (38). Recently, Prescott (24) described a hypothesis about a defect in cell-mediated immune mechanisms in foals that predisposes them to infection with a variety of intracellular pathogens, including *R. equi*. However, almost all clinical isolates from infected foals were virulent (35). This result is in contrast to those for the isolates from humans. Although limited information is available, it has been reported that *R. equi* is pathogenic not only in immunocompromised children but also in immunocompetent children (17).

In both animals and humans, it is believed that primary lesions develop in the lung following inhalation of soil-borne organisms (2, 23, 38). *R. equi* is widespread in domestic animals and their environments (2, 23). Our previous study (32) showed that soil at horse-breeding farms contained 10^2 to 10^4 CFU/g and that the feces of horses contained 10^2 to 10^5 CFU/g (wet weight). The prevalence of virulent *R. equi* strains among isolates from soil on farms with and without a history of *R. equi* infections ranged between 5 and 28% (32). The role of animals or soil in the transmission of *R. equi* to humans is unclear, but a history of exposure to farm animals or manure was reported in some cases in patients with and without AIDS (7, 9, 12). *R. equi* has also been recovered from soils with no history of exposure to animal feces (2). In a preliminary study, *R. equi* was frequently isolated from soil collected from areas free of animal contact in Japan (33). These results indicate that the patients without a history of exposure to farm animals or manure may have been exposed to *R. equi* in their environments. Because of the increasing number of AIDS cases worldwide and awareness of *R. equi* infection, it seems that *R. equi* will become a more prevalent pathogen in the future.

In the present study, the frequency of plasmid-positive isolates among the clinical isolates was 69.2% (27 of 39 isolates). During a survey of the prevalence of virulent *R. equi* in isolates from feces of horses and soil at horse-breeding farms with endemic infection, cryptic plasmids of various sizes were detected (30), as reported previously (36). However, the prevalence of cryptic plasmids was very low (<5%) (30). The cryptic plasmids among the clinical isolates in the present study were detected so frequently that these cryptic plasmids might have phenotypic characteristics of clinical importance, such as antibiotic resistance. Further studies will be done to characterize these plasmids by physical and genetic means.

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